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(54) Title: CODON AMIDITES AND METHOD OF USING THEM TO PRODUCE OLIGONUCLEOTIDES AND MUTAGENESIS LIBRARIES

(57) Abstract

Compounds of formula (1) or (2) wherein W, X, Y and Z are each independently hydrogen or a protecting group; B^1, B^2 and B^3 are each independently a base selected from the group consisting of adenine, guanine, cytosine, thymine and uracil; and ‖ is the residue of a ribose or deoxyribose, and methods for their synthesis and use are disclosed.

CODON AMIDITES AND METHOD OF USING THEM TO PRODUCE OLIGONUCLEOTIDES AND MUTAGENESIS LIBRARIES

Technical Field

The invention relates to the synthesis of various
5   types of oligonucleotides that are useful in analysis, therapy
and other applications. More specifically, the invention
concerns a technique for using preassembled 3'-phosphoramidite
trinucleotides as building blocks to make oligonucleotides
encoding a desired sequence of amino acids, optionally
10 containing positions with random amino acids. Randomized DNA
fragments, in particular, are useful in producing combinatorial
libraries of peptides or proteins with a variety of binding
properties, from which molecules of special interest can be
selected.

15   In one popular format, a DNA fragment is synthesized
corresponding to the sequence of a bacteriophage coat protein
with an appended "scrambled region" where all possible codons
will be presented. This fragment family is then inserted into
phage DNA such that, in an appropriate host cell population, a
20 library of different expressed peptide regions is created.
Selection of phage with desirable characteristics can then be
accomplished by a variety of criteria, and the sequence
responsible can be determined by sequencing the appropriate
portion of the phage DNA.

Background Art

25   Use of molecular biology techniques to encode
randomized sets of short peptides, of interest for their own
sake or as a means to modify specific sites within protein
domains, are useful to select molecules with desirable binding
30 specificities. Trinucleotides carrying certain protecting
groups have long been used as intermediates to synthesize
oligonucleotides corresponding to specific peptides of interest.
For example, see Wakabayashi et al., "Rapid Synthesis of
Oligodeoxynucleotides by using N-Methylimidazole as a
35 Condensation Catalyst. Syntheses of Dodecanucleotides
Corresponding to Complementary Deoxyribonucleic Acid of the

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Tetrapeptide Fragments of Cholecystokinin-Pancreozymin and
Vasoactive Intestinal Peptide", _Chem. Pharm. Bull._, _30_, 3951-58
(1982).

Certain dodecanucleotides have been synthesized on a
5 solid polymer support with phosphate triester methods by
elongation in the 3'-direction. The elongation can occur by
condensing the 3'-phosphodiester with 5'-deblocked dinucleotides
or trinucleotides having the 3'-(o-chlorophenyl)phosphoro-p-
anisidate moiety by using 1-(mesitylene sulfonyl)-3-nitro-1H-
10 1,2,4-tetrazole as the activating reagent. Ohtsuka et al.,
"Deoxyribonucleic Acids and Related Compounds. VIII. Solid-
Phase Synthesis of Deoxyribooligonucleotides with
3'-Modification by Elongation in the 3'-Direction", _Chem. Pharm._
Bull., _32_, 85-93 (1984).

15   When adapted to solid phase synthesis, the phosphate
triester method produces acceptable results for short fragments
(10-20 bases in length), but not for the longer fragments needed
to incorporate a scrambled sequence into a form suitable for
insertion into a vector. Triester methods have largely been
20 replaced by the phosphoramidite technique, which provides better
performance in the synthesis of long fragments. For example,
methods to increase the yield of long sequences prepared by
solid phase synthesis have been reported using
3'-phosphoramidite functionalized, protected nucleotide dimers.
25 Kumar et al., "Improvements in Oligodeoxyribonucleotide
Synthesis: Methyl N,N-Dialkylphosphoramidite Dimer Units for
Solid Support Phosphite Methodology", _J. Org. Chem._, _49_:25,
4905-4911 (1984).

Due to degeneracy in the DNA code, however, total
30 scrambling of all four nucleotides (adenine, guanine, cytosine
and thymine) at each site in a DNA sequence leads to more DNA
species than peptide species, with the excess growing
geometrically as a function of the length of the scrambled
sequence. For a hexapeptide, the 64 million possible
35 combinations of 20 amino acids expands to about 68 billion
possible combinations of encoding oligonucleotides. Further,
the inadvertent inclusion of stop codons sometimes results in

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the formation of truncated peptide sequences, which further
complicates the analysis.

    Two major approaches have been described to overcome
this redundancy problem. In a first approach, a semi-scrambled
5 set of nucleotides is used to take advantage of the third-
position redundancy in many codon assignments, for example, N-N-
(G/C), which symbolizes a trinucleotide having in the first and
second positions a random mix of all four nucleosides and, in
the third position, a mix of only guanine and cytosine. This
10 reduces the number of triplets from 64 to 32, while still
encoding all 20 amino acids, and has only one stop codon.

    However, each of the four nucleotides has a different
rate of incorporation onto the tip of the growing
oligonucleotide chain, so that the yield of the resulting
15 triplets can vary as much as five-fold. Accordingly, to obtain
a library containing all hexapeptides, the number of clones that
must be examined is more than 15,000 times larger than the
number indicated by simple combinatorial arithmetic.

    In the second approach, individual codons are
20 sequentially added to an oligonucleotide on a solid support,
usually resin beads, which have been divided into separate
containers. The contents of the containers are combined before
proceeding to the next codon, for which the resin beads holding
the growing chains are again separated into individual
25 containers. This resin splitting approach does, in principle,
produce a more nearly ideal final library, but requires a great
deal of synthetic effort. Huse, U.S. Patent No. 5,274,563
issued 23 November 1993; and Glaser et al., "Antibody
Engineering by Codon-based Mutagenesis in a Filamentous Phage
30 Vector System", J. of Immun., 149:12, 3903-13 (1992).
Specifically, handling problems, such as static electricity,
make quantitative physical transfer of the beads difficult, and
chemical inhomogeneity in the microscopically settled beads can
lead to poor coupling on some of the beads during solid phase
35 synthesis.

    Further, to obtain reasonably uniform representation
of each peptide in a hexapeptide library with this approach,
more than 64 million beads must be used to assure that the

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random pathway taken by each bead through the different containers does not leave out some sequence by mathematical chance. A 1 μM synthesis on controlled pore glass (CPG) beads is typical for synthesis of DNA used in most molecular biology
5 purposes. At this synthesis scale, however, only about 25,000 beads are present, which is too few to represent the entire library. Moreover, even if scale-up is attempted, it is a difficult procedure due to increasingly prohibitive handling problems and the attendant costs.

10 The Huse patent also speculates in passing that oligonucleotides could be synthesized from nucleotide triplets, but quickly discourages the use of this approach, stating that "the cost of synthesis from such triplets far exceeds that of synthesis from individual monomers because triplets are not
15 commercially available." (See, Huse, column 2, lines 18-29) Unexpectedly, the materials and methods of the invention provide a less labor-intensive procedure than that used in the art and, at the same time, provides increased confidence that all the desired sequences will be available, even on a modest synthetic
20 scale.

Disclosure of the Invention

The invention relates to compounds of the formula:
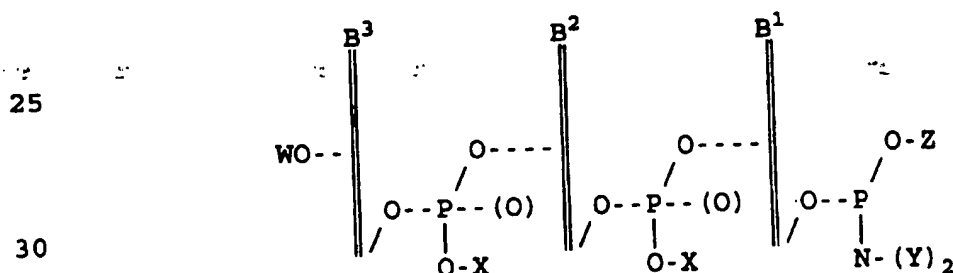
or
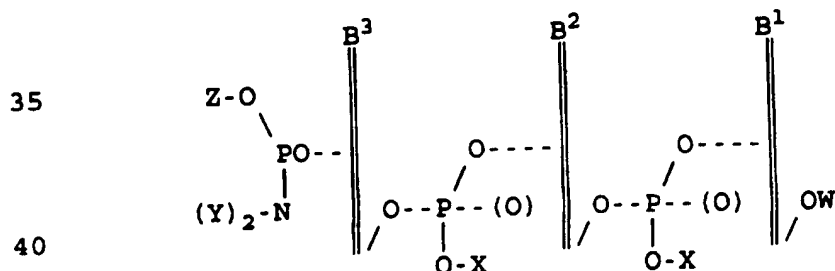
wherein W, Y and Z are each independently hydrogen or a
                protecting group;

   X is -CH$_2$CH$_2$CN;

   B^1, B^2 and B^3 are each independently a base selected
                from the group consisting of protected adenine,
5   protected guanine, protected cytosine, protected
                or unprotected thymine and protected or
                unprotected uracil; and

   ‖ is the residue of a ribose or deoxyribose.

10 Further, a method has been found for making these compounds and,
further, compounds wherein X may be hydrogen or any suitable
protecting group, comprising the steps of:

        a. treating a nucleoside comprising a base, a ribose
            or deoxyribose residue, and a first protecting
15   group at the 5′ position or the 3′ position, with
            a trialkylsilyl halide to produce the
            corresponding 3′- and 5′-substituted nucleoside;

        b. removing the first protecting group at the 5′ or
            3′ position to produce a 5′ or 3′ deprotected, 3′
20   or 5′-substituted nucleoside;

        c. coupling the 5′ or 3′ deprotected 3′ or 5′
            substituted nucleoside with a first nucleoside 3′
            or 5′ phosphoramidite and then oxidizing to form
            a phosphate triester;

25   d. deprotecting at the 5′- and 3′-termini to give
            the corresponding 3′,5′-dihydroxy dinucleoside;

        e. coupling the dihydroxy dinucleoside with a second
            nucleoside 3′ or 5′ phosphoramidite and oxidizing
            to produce the two corresponding 3′ or 5′ hydroxy
30   trinucleotides;

        f. separating away unwanted products; and

        g. converting the 3′- or 5′-hydroxy trinucleotide to
            a 3′- or 5′ phosphoramidite.

In another embodiment, a panel is made up of from two to twenty
35 of these 3′- or 5′-phosphoramidite trinucleotide codons or their
complements, where each codon encodes a different amino acid.

        Three processes for synthesizing an oligonucleotide
are disclosed. To synthesize an oligonucleotide encoding a

sequence of "n" pre-determined amino acids, or its complement,
the process of the invention comprises the steps of:

    (a)   coupling a trinucleotide codon, or its
           complement, onto a solid support;

5   (b)   sequentially condensing, through a
           phosphoramidite linkage, with an immediately
           preceding codon "n-1" trinucleotide codons or
           their complements, each codon or its complement
           corresponding to the next pre-determined amino
10   acid in the sequence; and

    (c)   cleaving the oligonucleotide from the solid
           support.

The automated solid phase supported chemistry used in the
invention can include a large number of materials generally
15 known to those of ordinary skill in the art. Examples of such
useful materials includes nucleoside-functionalized CPG, i.e.,
functionalized with deoxyribose cytosine (dC) or another
nucleoside, e.g., dG, dU or rC; tetrazole activator solution;
acetylating capping solutions; iodine oxidation solutions;
20 dichloroacetic acid deprotecting solvent; and automated DNA
synthesizers.

To synthesize an oligonucleotide encoding a peptide
having at least one pre-determined amino acid position and at
least one random amino acid position, a second synthetic process
25 of the invention comprises the steps of:

    (a)   coupling a first trinucleotide codon, or the
           complement to said codon, onto a nucleoside- or
           nucleotide-bearing solid support;

    (b)   for each pre-determined amino acid position,
           sequentially coupling through a phosphoramidite
30   to an immediately preceding codon, or complement,
           a trinucleotide codon, or its complement,
           corresponding to the pre-determined amino acid;

    (c)   for each random amino acid position, coupling to
           an immediately preceding codon or complement, a
35   mixture of from two to twenty trinucleotide
           codons, at least two of the trinucleotides

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representing a codon to a different amino acid;
and

(d)   cleaving said nucleotide from the solid support.

Steps (b) and (c) of this process are combined in such a way
5   that each pre-determined codon sequentially corresponds to each
pre-determined amino acid position in the oligonucleotide.

Further, the identity and ratio of codons used in each
such mixture can be made to represent the desired degree of
diversity in the corresponding random amino acid position.
10   Thus, if reduced coupling efficiency occurs with particular
codons making up the desired oligonucleotide, this defect can be
readily overcome by minor adjustments in the relative
concentrations of individual codons. For example, dC-
functionalized CPG or rC supports may be prone to give unequal
15   incorporation of bases when mixtures are used, perhaps due to
steric hindrance. With the invention, this inequality can be
compensated for by varying the relative concentrations of the
various codons.

To synthesize an oligonucleotide having at least one
20   region of random amino acid positions, the third synthetic
process of the invention comprises the steps of:

(a)   coupling at least one pre-existing trinucleotide
codon or complement onto a nucleoside- or
nucleotide-bearing solid support;

25   (b)   for each random amino acid position, sequentially
coupling through a phosphoramidite to the
immediately preceding codon, or complement, a
mixture of from two to twenty pre-existing
trinucleotide codons, each codon corresponding to
30   a different amino acid; and

(c)   cleaving the oligonucleotide from the solid
support.

In this process, the identity and ratio of codons used in each
such mixture are representative of the degree of diversity
35   desired in the corresponding random amino acid position.

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Brief Description of the Drawings

The present invention will be more clearly understood by referring to the following drawings, in which

Figure 1A shows a SAX HPLC trace of 16-mer
5   oligonucleotide dATACTTARCATGAGCC made with the codon amidites of the invention;

Figure 1B shows a SAX HPLC trace of the same 16-mer oligonucleotide dATACTTARCATGAGCC made with amidite monomers; and

10   Figure 1C shows a SAX HPLC trace of the result when both the fragment of Figure 1A and the fragment of Figure 1B are co-injected.

Figure 2 shows the results of analysis by reverse phase HPLC of coupling a codon amidite mixture to dC support.

15   ## Modes of Carrying Out the Invention

The compounds of the invention have the formula:

$$
\begin{array}{ccccc}
B^3 & & B^2 & & B^1 \\
| & & | & & | \\
WO- & O---- & & O---- & & O-Z \\
& / & & / & & / \\
O--P--(O) & & O--P--(O) & & O--P \\
/ \quad | & & / \quad | & & / \quad | \\
O-X & & O-X & & N-(Y)_2
\end{array}
$$

or

$$
\begin{array}{ccccc}
B^3 & & B^2 & & B^1 \\
Z-O & & | & & | \\
\backslash & & O---- & & O---- \\
PO-- & & / & & / \\
| & O--P--(O) & & O--P--(O) & & OW \\
(Y)_2-N & / \quad | & & / \quad | & & / \\
& O-X & & O-X
\end{array}
$$
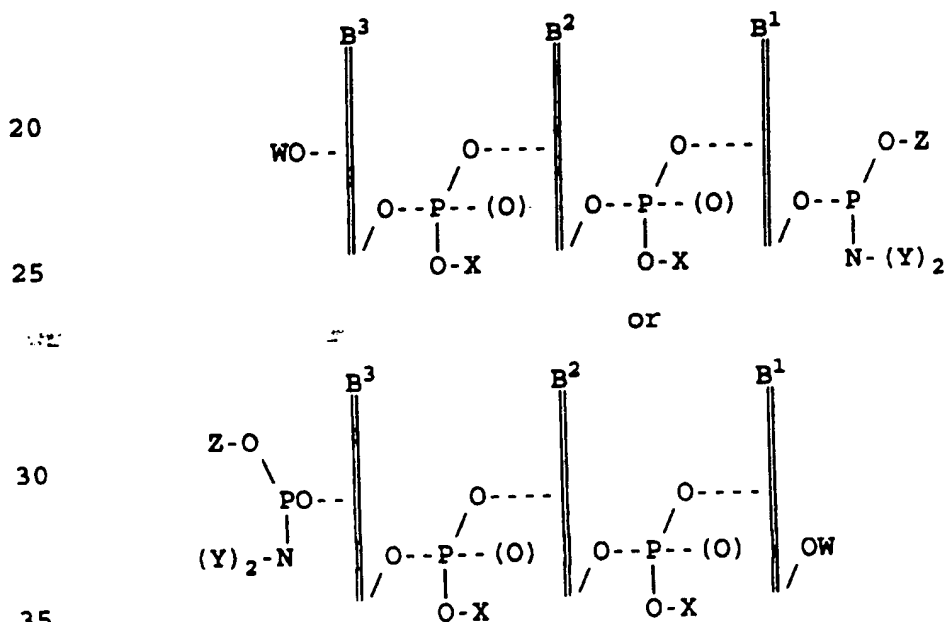
as shown and described above.

W, X, Y and Z in the above formula are each independently hydrogen or a protecting group. Preferably, the protecting group is selected such that the reaction between the
40   protecting group moiety and the atom or atoms at the position

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being protected is a high-yield step. Further, all of the
protecting groups used should be stable to the usual reaction
conditions, such as are used in condensation reactions, to
further manipulate the protected compound.

5   While a protecting group at the 3'-position may
desirably be capable of selective cleavage without detrimental
effect on a protecting group in the 5'-position, it should be
noted that this is not always a necessary feature of the process
of the invention. Specifically, in the process of the

10 invention, both the 3'- and the 5'-positions of the dimer
intermediates are deprotected, usually simultaneously.
Subsequent reaction forms mixtures from which unwanted
components are removed.

W can be hydrogen or any one of a wide variety of

15 protecting groups so long as they can be removed independently
of the other protective groups (X, Y and Z). Preferably, when W
is a protecting group, it is a triphenylmethyl group, such as
DMT (dimethoxytrityl) or monomethoxytrityl; a carbonyl-
containing group such as FMOC (9-fluorenylmethyloxycarbonyl) or

20 levulinoyl; an acid-cleavable group such as pixyl; a fluoride-
cleavable alkylsilyl group such as t-BDMSi (tert-butyl
dimethylsilyl), triisopropyl silyl, or trimethylsilyl; and the
like. Most preferably, W is the protecting group DMT.

X may be H or any one of a wide variety of protecting

25 groups, so long as it can be removed without destroying the
product nucleotide. For example, X may be an alkyl group, such
as methyl, ethyl, isopropyl, tert-butyl, or n-hexyl; haloalkyl
such as haloethyl; cyanoalkyl such as $-CH_2CH_2CN$; an aryl group
such as o-chlorophenyl or methoxyphenyl; and the like. However,

30 most preferably, X is the cyanoalkyl group $-CH_2CH_2CN$.

Y may be H or any one of a wide variety of groups.
However, as a guideline to selecting useful groups, Y should
preferably be a hydrocarbon. For example, useful Y groups
include alkyl groups such as methyl, ethyl, isopropyl, tert-

35 butyl, or n-hexyl. Alternatively, two Y groups, taken together,
may form a heterocyclic ring with the nitrogen atom protected,
such as morpholino, piperidino, pyrollidino, and the like. Most
preferably, Y is an alkyl group, such as methyl or isopropyl.

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Z can be any one of large number of different
protecting groups and should be chosen so as to have the same
characteristics as X.   Suitable examples of Z as a protecting
group include an alkyl group, such as methyl, ethyl, isopropyl,
tert-butyl, or n-hexyl; a cyanoalkyl group such as $-CH_2CH_2CN$; an
aryl group such as o-chlorophenyl, or methoxyphenyl; and the
like.   Preferably, Z is cyanoalkyl such as $-CH_2CH_2CN$.

In a particularly preferred embodiment, W is DMT, X
and Z are each $-CH_2CH_2CN$, and Y is an isopropyl group.   Methods
of manipulating various protective groups with respect to DNA
are known to those of ordinary skill in the art.   Using similar
methods to protect the 2'-hydroxy group of an RNA molecule with
a protecting group is also known, e.g., see Wang et al.,
"Enzymatic and NMR Analysis of Oligoribonucleotides Synthesized
with 2'-tert-Butyldimethylsilyl Protected
Cyanoethylphosphoramidite Monomers", Nucleic Acids Research,
18:11, 3347-52 (1990).

The bases B^1, B^2 and B^3 are each independently selected
from the group consisting of protected adenine, protected
guanine, protected cytosine, protected or unprotected thymine
and protected or unprotected uracil.   When any one or more of
B^1, B^2 and B^3 is adenine, cytosine or guanine, it should be
protected with a group such as benzoyl, isobutyryl,
phenoxyacetyl, methoxyacetyl, an amidine, or the like.

In a particularly preferred embodiment, B^1 is selected
from the group consisting of an adenine base protected with a
benzoyl protecting group, a thymine base, a cytosine base
protected with a benzoyl protecting group, and a guanine base
protected with an isobutyryl protecting group.   In another
preferred embodiment, B^2 is selected from the group consisting
of thymine and guanine and, when B^2 is guanine, it is protected
with an isobutyryl protecting group.

In yet another preferred embodiment, B^3 is selected
from the group consisting of protected adenine and cytosine.
When B^3 is either adenine or cytosine, it is preferably
protected with a benzoyl protecting group.

Preferred groupings of B^1, B^2 and B^3, with protective
groups often preferred for each grouping, are shown below:

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$$B^3 = A^{bz}; \quad B^2 = T; \text{ and } B^1 = A^{bz}$$
$$B^3 = T; \quad B^2 = T; \text{ and } B^1 = C^{bz}$$
$$B^3 = C^{bz}; \quad B^2 = T; \text{ and } B^1 = A^{bz}$$
$$B^3 = G^{ibu}; \quad B^2 = T; \text{ and } B^1 = A^{bz}$$
$$B^3 = C^{bz}; \quad B^2 = G^{ibu}; \text{ and } B^1 = A^{bz}$$

"‖" in the above formula represents the residue of a ribose or deoxyribose. Ribose is the pentose $CH_2OH(CHOH)_3CHO$ that forms the sugar backbone of an RNA polynucleotide chain in its furanose form by a series of 5'-3' sugar-phosphate links. Deoxyribose has one less hydroxy group at the 2-position of the sugar ring and is the sugar that forms the backbone of a DNA chain.

The compounds of the invention can be made by:

a. treating a nucleoside comprising a base, a ribose or deoxyribose residue, and a first protecting group at the 5' position or the 3' position, with a trialkylsilyl halide to produce the corresponding 3'- and 5'-substituted nucleoside;

b. removing the first protecting group at the 5' or 3' position to produce a 5' or 3' deprotected, 3' or 5'-substituted nucleoside;

c. coupling the 5' or 3' deprotected 3' or 5' substituted nucleoside with a first nucleoside 3' or 5' phosphoramidite and then oxidizing to form a phosphate triester;

d. deprotecting at the 5'- and 3'-termini to give the corresponding 3',5'-dihydroxy dinucleoside;

e. coupling the dihydroxy dinucleoside with a second nucleoside 3' or 5' phosphoramidite and oxidizing to produce the two corresponding 3' or 5' hydroxy trinucleotides;

f. separating away unwanted products; and

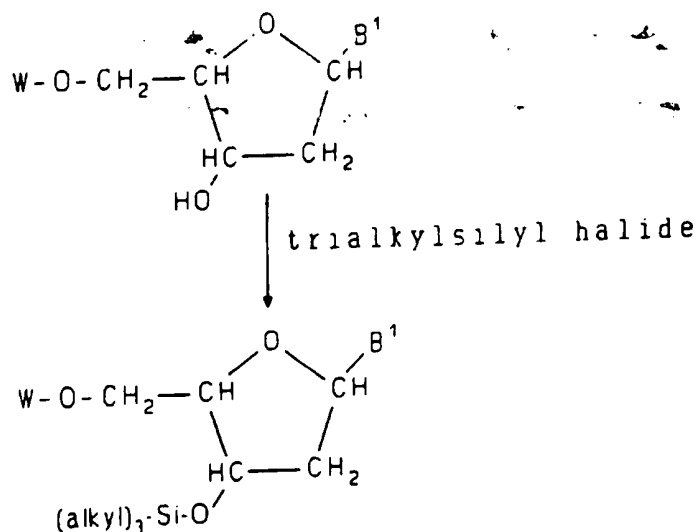g. converting the 3'- or 5'-hydroxy trinucleotide to a 3'- or 5' phosphoramidite.

Step "a." of this particularly advantageous process for making the compounds of the invention comprises treating the starting nucleoside, which has a 3'- or 5'-protecting group such

as DMT, with a trialkylsilyl halide, such as t-butyl
dimethylsilane halide. For example, when the starting
nucleoside has a 5'-protecting group, the product will be a
3'-trialkylsilyl, 5'-protected nucleoside. This reaction is
5   illustrated below:

$$W-O-CH_2-CH \overset{O}{\underset{HC-CH_2}{\diagdown}} \overset{B^1}{CH}$$
$$HO$$

trialkylsilyl halide

$$W-O-CH_2-CH \overset{O}{\underset{HC-CH_2}{\diagdown}} \overset{B^1}{CH}$$
$$(alkyl)_3 \cdot Si-O$$

where W and B_1 are as defined above.

This reaction is typically carried out in the presence
of $AgNO_3$ or imidazole, which acts as a catalyst, and an organic
solvent, such as pyridine or tetrahydrofuran. Preferably, the
10   reaction takes place in an inert atmosphere, such as that
provided by nitrogen or argon gas. The product may be isolated
from the rest of the reaction mixture by any convenient method,
such as by drowning out in a non-solvent, precipitating out,
extraction with an immiscible liquid, evaporation of a solvent,
15   or some combination of these or other methods. A particularly
preferred method of adding a 3'-t-butyl dimethylsilyl protecting
group is provided by Ogilvie et al., _Pure and Appl. Chem._, _59_,
325-30 (1987).

In step "b.", the protecting group at the 5'- or
20   3'-position can be removed selectively by any one of a number of
various procedures, for example, by treatment with a mineral
acid, such as HCl or H_2SO_4; an organic acid such as HOAc,
dichloroacetic acid, trichloroacetic acid, benzenesulfonic acid;
another strong acid; a metal halide such as $ZnBr_2$ or another

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Lewis acid; a base such as piperadine or hydrazine; and the like.

In the removing step "b.", a solvent is typically used, such as an alcohol, acetonitrile, diethyl ether, acetone,
5   ethyl acetate, or the like. Preferably, the solvent is acetonitrile.

The 3'- or 5'-deprotected product may be isolated from the rest of the reaction mixture by any convenient method, such as by drowning out in a non-solvent, precipitating out,
10 extraction with an immiscible liquid, evaporation of a solvent, or some combination of these or other methods. Preferably, the 3-silyl-protected nucleosides are isolated by crystallization.

Step "c." of the above-described process involves coupling the 5'- or 3'-deprotected, 3'- or 5'-substituted
15 nucleoside with a first nucleoside phosphoramidite, followed by oxidation of the internucleotide trivalent phosphorous to form a phosphate triester. The first nucleoside phosphoramidite has the formula:

$$W-O-CH_2-CH \begin{array}{c} O \\ \diagup \quad \diagdown \end{array} CH-B^2$$
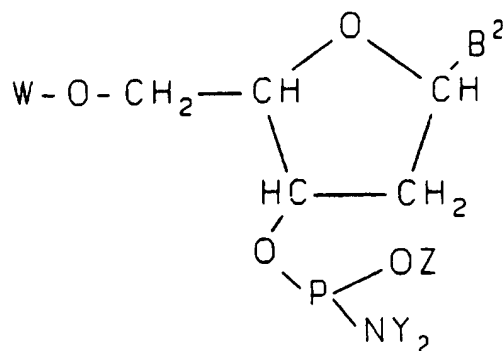
where W, Y, Z and B^2 are as defined above. Such
20 phosphoramidites are usually available commercially under the trade name "DNA Amidites." Typically, the nucleoside phosphoramidite is used in the amount of from about 1.0 to about 1.1 equivalents, most preferably about 1.1 equivalents.

After being condensed with, for example, the 5'-
25 deprotected and 3'-substituted nucleoside described above, and after the dinucleotide phosphite triesters have been oxidized to phosphate triesters, the coupled product has the formula:

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$$W \cdot O \cdot CH_2 \underset{\overline{}}{} CH \overset{\displaystyle O}{\diagup\diagdown} CH \overset{B^2}{}$$
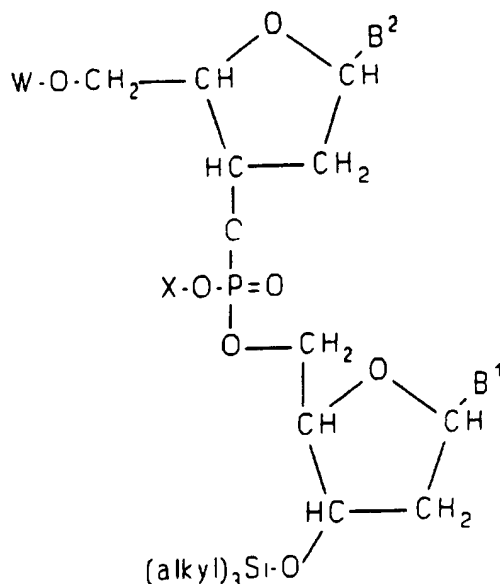
where W, X, B^1 and B^2 are as defined above.

This dimer formation reaction typically takes place in the presence of a tetrazole for the purpose of activating the phosphoramidite. The amount of tetrazole present can vary
5   widely between about 5 to about 10 equivalents, but preferably is about 10 equivalents. Other materials that can be substituted for tetrazole include benzotriazole and p-nitrophenyl tetrazole.

The dinucleotides are then given a deblocking
10 procedure, such as treatment with acid, to deprotect the 5'- and 3'-termini, using one of the deblocking procedures described above, either alone or in combination with each other or other procedures known to those of ordinary skill in the art. The resulting deprotected 3',5'-dihydroxy dinucleoside is typically
15 isolated from the rest of the reaction mixture by any convenient method, such as by drowning out in a non-solvent, precipitating out, extraction with an immiscible liquid, evaporation of a solvent, or some combination of these or other methods. A preferred isolation method is by chromatography.

20   Further, when a silyl protecting group is present in step "d.", a silyl cleaving agent such as KF is also typically added to the reaction mixture, primarily to diminish side reactions but also to accelerate the deprotecting reaction.

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Other examples of such agents include tetrabutylammonium
fluoride.

Next, in step "e.", the deprotected 3',5'-dihydroxy
dimer is coupled with a second nucleoside phosphoramidite having
5   a base B^3, and the trimer product is then oxidized to produce
the two corresponding hydroxy trinucleotides. In a preferred
embodiment, the deprotected dimer is coupled with the second
phosphoramidite to produce the corresponding 5'-W-3'-hydroxy
trinucleotide. The resulting 5'-W-3'-hydroxy trinucleotide can
10   then be isolated, as in step "e." by any means for separating
away unwanted products, preferably by column chromatography.
The final step, "g.", comprises converting the 3'- or 5'-hydroxy
trinucleotide, such as 5'-W-3'-hydroxy trinucleotide, to the
corresponding 3'- or 5-'phosphoramidite, preferably by the
15   procedure of Sinha et al., Tett. Lett., 24, 5843-46 (1983), and
isolating this by column chromatography.

The resulting 3'- or 5'-phosphoramidite
trinucleotides, also called codon amidites, can be coupled onto
nucleoside or nucleotide-bearing solid supports with semi-manual
20   methods. For example, a Biosearch 8750 four-column, automated
DNA synthesizer running a synthesis program without the coupling
step can be used for 5'-deblocking, oxidation and capping, as
well as washing between steps.

Further, a mixture of from two to twenty 3'- or 5'-
25   phosphoramidite trinucleotide codons, or their complements, can
be used to assemble a panel of codons, with each codon encoding
a different amino acid. Such panels are useful for making
oligomers, such as synthetic DNA fragments, by sequentially
coupling the individual codons in the panel to a dC nucleoside-
30   or nucleotide-functionalized support.

Specifically, in accordance with one of the processes
of the invention, an oligonucleotide can be synthesized to
encode a sequence of "n" pre-determined amino acids, or its
complement. Such a process comprises the steps of:

35   (a)   coupling a trinucleotide codon or its complement
                  onto a solid support;
            (b)   sequentially condensing, through a
                  phosphoramidite linkage, with each immediately

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preceding codon, or complement, n-1 trinucleotide
codons or their complements, each codon or its
complement corresponding to the next pre-
determined amino acid in the sequence; and

5   (c) cleaving the oligonucleotide from the solid
support.

Step (b) above, directed to sequential condensation, is as
described above generally for condensation reactions. Step (a),
the coupling of a codon onto a solid support, and step (c), the
10 cleavage of the resulting oligonucleotide from the support, are
performed by methods known to those of ordinary skill in the
art. The cleavage procedure usually removes all protecting
groups simultaneously to generate biologically active material.

An oligonucleotide encoding a peptide having at least
15 one pre-determined amino acid position and at least one random
amino acid position can be prepared in accordance with the
invention by a process comprising the steps of:

(a) coupling a first trinucleotide codon, or the
complement to said codon, onto a nucleoside- or
20   nucleotide-bearing solid support;

(b) for each pre-determined amino acid position,
sequentially coupling through a phosphoramidite
to an immediately preceding codon, or complement,
a trinucleotide codon, or its complement,
25   corresponding to the pre-determined amino acid;

(c) for each random amino acid position, coupling
through a phosphoramidite to an immediately
preceding codon, or complement, at least two of
the trinucleotides representing a codon, at least
30   two of the trinucleotides representing a codon to
a different amino acid; and

(d) cleaving said nucleotide from the solid support.

Each pre-determined codon sequentially corresponds to each pre-
determined amino acid position in the desired oligonucleotide.
35 Further, steps (b) and (c) above are preferably combined in such
a way that the identity and ratio of the codons used in each
said mixture represent the degree of diversity desired in the
corresponding random amino acid position.

Finally, in accordance with a third embodiment of the invention, an oligonucleotide having at least one region of random amino acid positions, which may be optionally preceded or followed by "n" pre-determined flanking amino acid sequences, or
5   their complements, the third synthetic process of the invention comprises the steps of:

(a)   coupling at least one pre-existing trinucleotide codon, or complement, onto a nucleoside- or nucleotide-bearing solid support;

10 (b)   for each random amino acid position, sequentially coupling through a phosphoramidite to the immediately preceding codon, or complement, a mixture of from two to twenty pre-existing 3'-phosphoramidite trinucleotide codons, each
15   codon corresponding to a different amino acid; and

(c)   cleaving the oligonucleotide from the solid support.

The identity and ratio of codons used in each mixture represent
20 the degree of diversity desired in the corresponding random amino acid position.

These processes of the invention for making oligonucleotides provide new methods for building diverse DNA libraries, using the pre-formed triplet codons in combination
25 with reliable phosphoramidite DNA synthesis. Although coupling efficiencies may be modest, the amplification power of the polymerase chain reaction (PCR) renders them adequate for a wide range of purposes. For example, combinatorial libraries using a systematically diversified sets of monomers have been made by
30 chemical means. Using the codon amidites of the invention, the advantages of a practical synthetic pathway and the ability to thoroughly characterize the resulting compounds can now be extended to recombinant libraries. Selected subsets of amino acids can be used to analyze structure/function relationships in
35 a wide variety of contexts, such as the role of steric bulk versus electronic properties in a particular chemical environment. Specifically, the striking biases that exist in amino acids present at short distances from bound ligands have

already been noted in a collection of about 50 protein crystal
structures, thus enabling the synthesis of novel binding sites
by directed mutagenesis. This, in turn, results in the ability
to produce more selectively targeted biological materials such
5 as drugs and diagnostic markers.

      It is therefore seen that the compositions and
processes of the invention are superior to the generation of
codons using mixtures of monomers to obtain a random mix of
amino acids in a particular position, because (1) stop codons
10 are eliminated; (2) the codons expressed are more easily
controlled, particularly when the expression of a library
containing less than 20 amino acids at a given site is desired;
and (3) the codon redundancy inherent in the standard N-N-(G/C)
mixed site is eliminated since an inflated library size is not
15 needed to achieve complete representation. Moreover, the
compositions and processes of the invention are clearly superior
to the physical support-splitting methods, which involve
repetitive coupling, mixing, and dividing steps, since less
labor is required. Further, only a modest synthesis scale is
20 needed to provide all of the desired sequences.

      The following examples are intended to illustrate but
not to limit the invention.

Example 1:   Preparation of N^4-Benzoyl-3'-tert-butyl-
                 dimethylsilyl Deoxycytidine
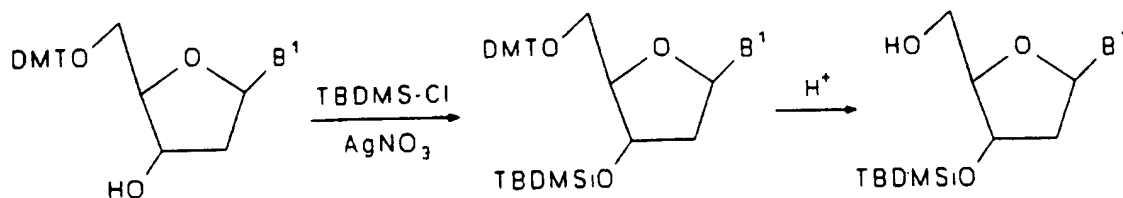
25 where B^1 is C^{bz}.

      A solution of 5 g (7.9 mmol) N^4-benzoyl-5'-O-(4,4'-
dimethoxytrityl)-2'-deoxycytidine in 200 mL of pyridine was
reduced to a foam in vacuo. Another 200 mL of pyridine and 2.5
g of $AgNO_3$ was added, and about 50 mL of the pyridine solvent
30 was removed by rotary evaporation. The flask containing the
reaction mixture was flushed with argon, and 3 g of the solid

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tert-butyldimethylsilyl chloride (TBDMS-Cl) (19.8 mmol) was
added. The resulting solution was allowed to stand overnight.
        The next day, 10 mL of methanol was added, and the
reaction mixture was reduced to a solid *in vacuo*. 200 mL of
5   ethyl acetate and 100 mL of water were added, and the mixture
was transferred to a separatory funnel and allowed to separate.
After separating the organic layer was washed with 100 mL of
water and dried over anhydrous Na_2SO_4. The solution was reduced
to a foam and subjected to high vacuum overnight. The yield was
10 5.8 g (98%) of a single spot material (rf 0.81 in 9:1
CH_2Cl_2:MeOH).

        This 3'-protected product was dissolved in 200 mL
CH_3CN, and 10 mL of 6N HCl was added. The reaction mixture
turned red, was allowed to stand for one minute, and then was
15 neutralized to a colorless endpoint by adding 28% aqueous
ammonia. The solution was reduced to a solid *in vacuo*. The
solid was dissolved in 200 mL of methanol. When 100 mL of water
were added, the solution became cloudy. The mixture was placed
in a separatory funnel and washed with 200 mL of hexane.
20 Crystals formed in the aqueous layer, and these were collected
by filtration and washed with hexane. The aqueous layer was
then chilled, and the resulting crystals were combined with
those previously obtained to give 2.5 g of white powder (73%
yield; rf 0.64; mp 105-110°C).

25 ^1H NMR (300 mHz, $CDCl_3$): δ 8.3 (d, 1H); 7.8 (d, 2H); 7.5 (d,
            2H); 7.4 (5, 3H); 7.2 (s, 1H); 6.15 (s, 1H); 4.45 (m,
            1H); 3.95 (s, 2H); 3.75 (s, 2H); 2.4 (m, 1H); 2.3 (m,
            1H); 0.8 (s, 9H); 0.0 (s, 6H).
m/z 469.7, calculated. MNa^+ 469.7.

Example 2:   Preparation of Thymidine-3'-yl N⁴-Benzoyl-
              deoxycytidine-5'-yl Cyanoethylphosphate
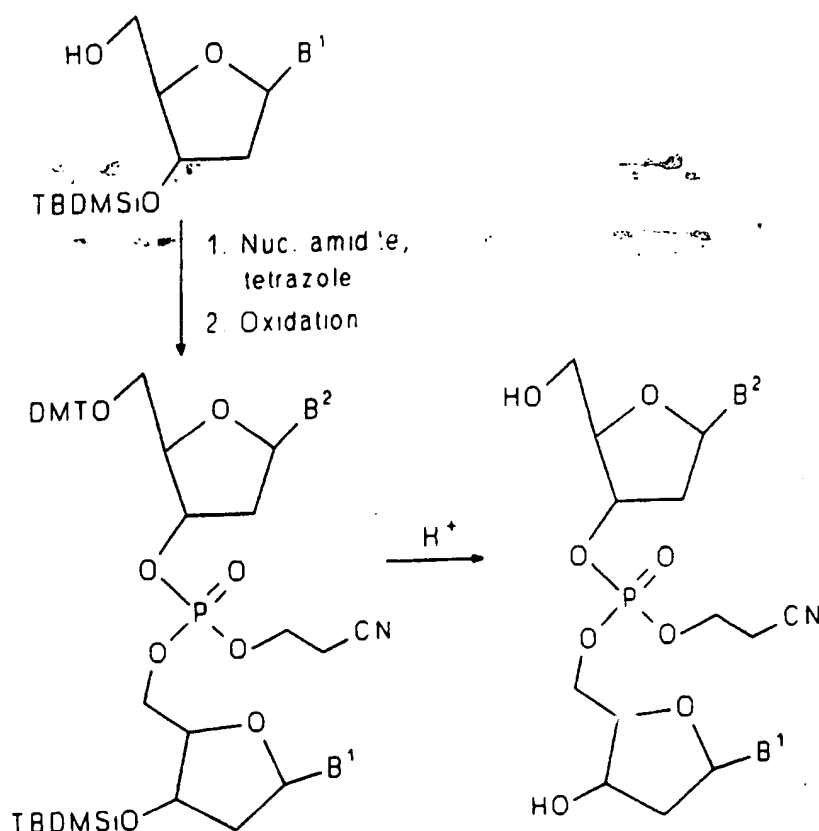
where B^1 is C^{bz} and B^2 is T.

A solution of 1 g (2.2 mmol) N^4-benzoyl-3'-tert-
butyldimethylsilyl deoxycytidine in 150 mL of dry CH_3CN was
reduced to a foam in vacuo. The procedure was repeated, and the
residue was subjected to high vacuum for one hour. About 40 mL
of dry CH_3CN was added and, after the solid starting material
dissolved, 2 g (2.7 mmol) of 5'-O-(4,4'-
dimethoxytrityl)thymidine-3'-O-(2-cyanoethyl-N,N-
diisopropylphosphoramidite was added. To this was added 10 mL
of 0.5 N tetrazole in dry CH_3CN, and the mixture was allowed to
stand for 15 minutes. 150 mL of ethyl acetate, 50 mL of water,
100 µL of pyridine, and 2 g of iodine were added, and the
mixture was shaken until an orange color persisted. To this was
added 4 g of $Na_2S_2O_3$, and the mixture was shaken in a separatory
funnel until the orange color disappeared. The layers were
separated, and the organic phase was washed with 200 mL of a

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saturated solution of NaHCO$_3$ in water and dried over anhydrous
Na$_2$SO$_4$. The solution was filtered and reduced to a foam *in
vacuo*. The yield was 2.7 g (100%; rf 0.6 in 10% MeOH/CH$_2$Cl$_2$).
H.R.M.S. calculated for C$_{56}$H$_{66}$N$_6$C$_{14}$SiP: 1105.4144.
5 Found: 1105.4164.

          This product was dissolved in 100 mL CH$_3$CN, and 10 mL
of 6N HCl were added. The reaction mixture turned orange and
was allowed to stand for 90 minutes. Cautiously, 28% aqueous
ammonia was added until the orange color disappeared. 5 mL of
10 pyridine was then added. The solution was reduced to a solid *in
vacuo* and subjected to a high vacuum overnight. The solid was
partially dissolved in 40 mL of 5% MeOH/CH$_2$Cl$_2$ and applied to a
bed of silica (4 X 15 cm) packed with the same solvent. Elution
proceeded with a series of MeOH/CH$_2$Cl$_2$ mixtures, as follows: 500
15 mL of 5% MeOH, 500 mL of 10%, 500 mL of 15%, and 500 mL of 20%
MeOH/CH$_2$Cl$_2$. The product-containing fractions eluted at 20%
MeOH/CH$_2$Cl$_2$, were pooled, and then reduced *in vacuo* to give,
after high vacuum overnight, 1.2 g of a clear tar (68% yield; rf
0.19 at 10% MeOH/CH$_2$Cl$_2$).

20 MALDI m/z 711.6, calculated. MNa$^+$ 711.
^{1}H NMR (400 mHz, DMSO D$_6$): δ 11.2 (s, 1H); 11.1 (m, 1H); 8.1 (d,
          1H); 7.9 (d, 2H); 7.6 (s, 1H); 7.5 (t, 1H); 7.4 (t,
          2H); 7.2 (s, 1H); 6.1 (m, 2H); 5.4 (d, 1H); 5.1 (m,
          1H); 4.9 (m, 1H); 4.1 (m, 5H); 3.9 (m, 2H); 3.5 (m,
25   2H); 2.8 (5, 2H); 2.3 (m, 3H); 2.0 (m, 1H).

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Example 3: Preparation of N⁶-Benzoyl-5'-O-(4,4'-di-
methoxytrityl)deoxyadenosine-3'-yl Cyano-
ethylphosphate-5'-yl Thymidine-3'-yl
N4-Benzoyldeoxycytidine-5'-yl Cyano-
ethylphosphate
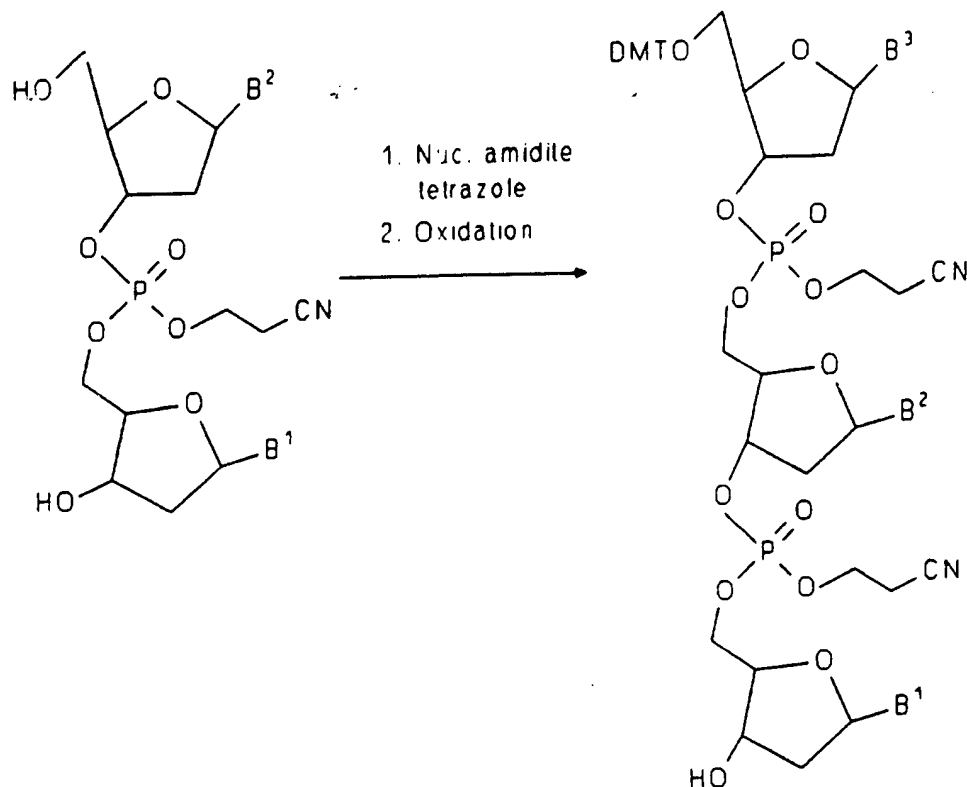
where B¹ is C^{bz}, B² is T, and B³ is A^{bz}.

A solution of 1.2 g (1.4 mmol) of thymidine-3'-yl N⁴-
benzoyldeoxycytidine-5'-yl cyanoethylphosphate in a mixture of
100 mL dry CH_3CN and 10 mL DMSO was reduced to a volume of about
10 mL *in vacuo*. This process was repeated with 100 mL of dry
CH_3CN.

After flushing with argon, 60 mL dry CH_3CN and 10 ml
of 0.5 N tetrazole in CH_3CN were added. Three aliquots of 500
mg N⁶-benzoyl-5'-O-(4,4'-dimethoxytrityl)-deoxyadenosine-3'-
O-(2-cyanoethyl)-N,N-diisopropyl-phosphoramidite were added at
10 minute intervals (total 1.5 g, 1.7 mmol). After 10 minutes,
150 mL of ethyl acetate and 50 mL of water were added, along
with 100 μL of pyridine. About 1 g of iodine was then added,
and the flask was shaken until an orange color persisted. To

this was added 4 g of $Na_2S_2O_3$, and the mixture was shaken until
the orange color disappeared. The layers were separated, and
the organic phase was washed with 200 mL of saturated $NaHCO_3$
solution and dried over anhydrous Na_2SO_4. The mixture was

5   filtered, and the filtrate was reduced to a foam in vacuo. A
silica column (3 x 12 cm) was prepared using 2% methanol and 1%
pyridine in CH_2Cl_2. The trimer product was applied to the column
using 20 mL of the same solvent. Elution proceeded with a
series of $MeOH/CH_2Cl_2$ mixtures, specifically, 200 ml of 2% MeOH,

10   200 ml of 4%, 200 mL 6%, 200 mL 8%, 200 mL 10%, 200 mL 12%, and
200 mL of 14% $MeOH/CH_2Cl_2$. The product-bearing fractions, which
eluted at 14% $MeOH/CH_2Cl_2$, were pooled and reduced in vacuo to
give 600 mg (29% yield) of the desired trimer (rf 0.28 in 10%
$MeOH/CH_2Cl_2$).

15   MALDI m/z 1492, calc'd. MNa^+ 1489.
^1H NMR (400 mHz, $CDCl_3$): δ 8.6 (d, 1H); 8.2 (d, 1H); 8.03 (t,
          1H); 8.0 (d, 2H); 7.8 (t, 2H); 7.6-7.1 (m, 17H); 6.8
          (m, 4H); 6.5 (t, 1H); 6.1 (s, 1H); 6.0 (m, 1H); 5.3
          (broad s, 1H); 5.2 (s, 1H); 5.0 (broad s, 1H); 4.5-4.2

20   (m, 15H); 3.8 (s, 6H); 3.4 (m, 2H); 3.2 (m, 1H);
          2.8-2.2 (m, 12H); 1.8 (m, 3H).

Example 4:   Preparation of N^6-Benzoyl-5'-0-(4,4'-di-
methoxytrityl)deoxyadenosine-3'-yl Cyano-
ethylphosphate-5'-yl Thymidine-3'-yl
N^4-Benzoyldeoxycytidine-5'-yl Cyanoethyl-
phosphate-3-0-(2-cyanoethyl)-N,N-diiso-
propylphosphoramidite
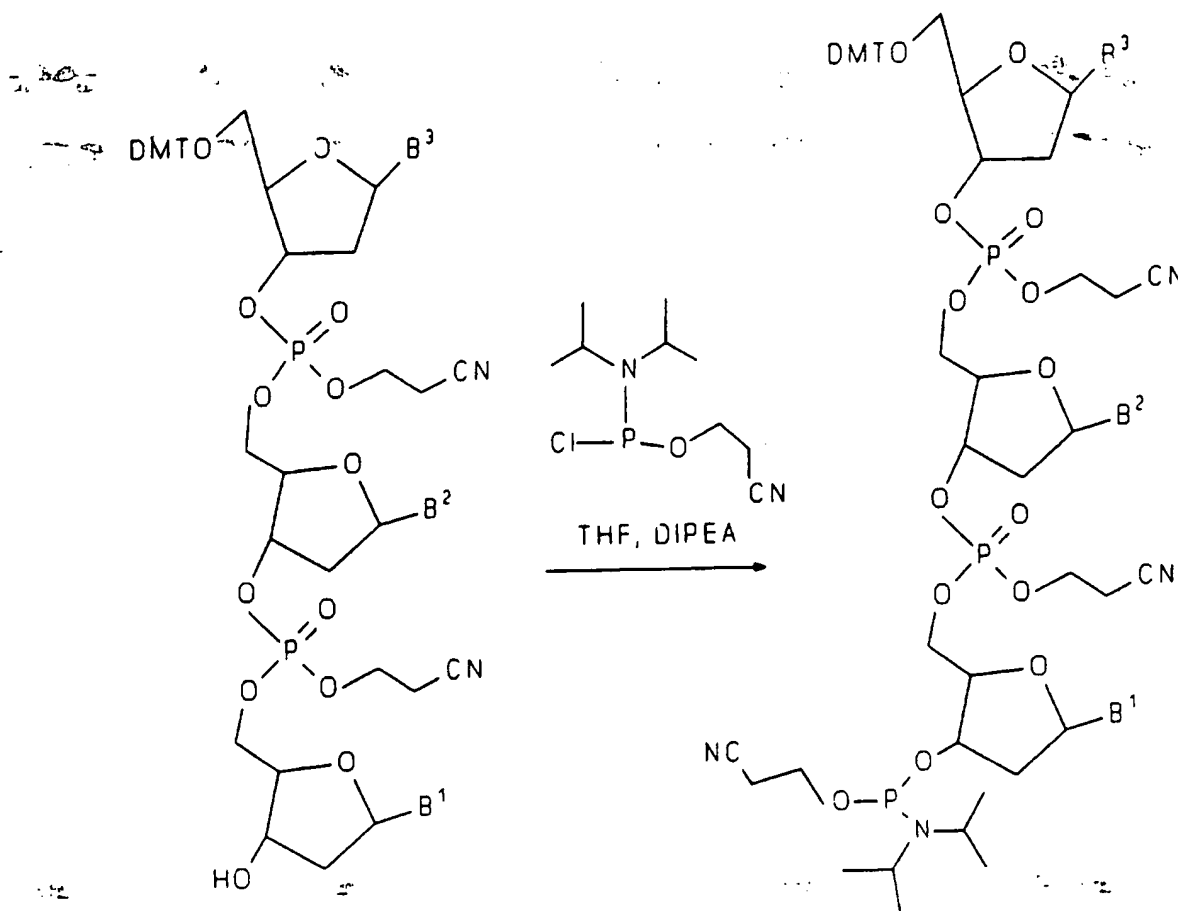
where B^1 is C^{bz}, B^2 is T, and B^3 is A^{bz}.

A mixture of 50 mL of CH_3CN and 100 ml THF were used
to dissolve 400 mg (0.27 mmol) of N^6-benzoyl-
5'-0-(4,4'-dimethoxytrityl)deoxyadenosine-3'-yl
cyanoethylphosphate-5'-yl thymidine-3'-yl
N^4-benzoyldeoxycytidine-5'-yl cyanoethylphosphate (from Example
3). The solution was stripped to a solid *in vacuo*. After argon
was blown into the flask, the solid was re-dissolved in 60 mL
THF. About 1 mL of diiso-propylethylamine (DIPEA) was added,
along with 300 μL 2cyanoethyl-
N,N-diisopropylchlorophosphoramidite (318 mg, 1.3 mm). The

solution was allowed to stand for 20 minutes, and 5 mL of 5%
aqueous NaHCO$_3$ were added. The solvent was removed *in vacuo*,
and 100 mL of ethyl acetate was added. The solution was washed
with 60 mL of a 5% aqueous solution of NaHCO$_3$, dried over Na$_2$SO$_4$,

5   and reduced to a solid *in vacuo*. A silica column (2 X 7 cm) was
prepared using 2% methanol and 1% pyridine in CH$_2$Cl$_2$. The crude
product was applied to the column using 10 mL of this solvent.
Elution proceeded with a series of MeOH/CH$_2$Cl$_2$ mixtures,
specifically, 100 mL of 2% MeOH/CH$_2$Cl$_2$, 100 mL of 3%, 100 mL 4%,

10 100 mL 5% and 100 mL of 6% MeOH/CH$_2$Cl$_2$. The product-bearing
fractions, which eluted at 6% MeOH/CH$_2$Cl$_2$, were pooled and
reduced *in vacuo* to give 140 mg (31% yield) of the desired codon
amidite (rf 0.45 at 10% MeOH/CH$_2$Cl$_2$).

^{31}P NMR (162 mHz, CDCl$_3$): δ δ 147.2, 147.16, (P^{III}), -4.560,

15   -5.563 (P^V). Area ratio P^{III} / P^V = 20.243 / 39.782.
Anal. Calc'd for C$_{82}$H$_{97}$N$_{14}$O$_{20}$P$_3$·CH$_2$Cl$_2$: C, 56.24; H, 5.58; N,
11.19. Found: C, 55.91; H, 5.23; N, 11.32.

Example 5:   Preparation of Other Codon
                  Phosphoramidite Compounds

20   The following additional trinucleotide codon
phosphoramidites made by the general methods described above in
Examples 1-4.

25
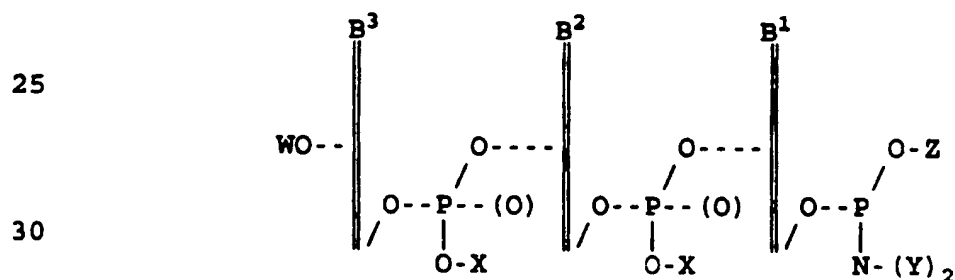
wherein W is DMT, X and Z are both -CH$_2$CH$_2$CN, and Y is
                diisopropyl amine;
        ‖ is the residue of a ribose or deoxyribose; and

35   B^1, B^2 and B^3 for Compounds 1-4 are as shown below:
        Compound 1:   B^3 = A^{bz}; B^2 = T; and B^1 = A^{bz}
        Compound 2:   B^3 = T; B^2 = T; and B^1 = C^{bz}

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Compound 3: $B^3 = G^{ibu}$; $B^2 = T$; and $B^1 = A^{bz}$

Compound 4: $B^3 = C^{bz}$; $B^2 = G^{ibu}$; and $B^1 = A^{bz}$

Example 6:   DNA Synthesis and Analysis

The codon amidite triplets dATA, dCTT, dATC, dATG and
5   dAGC were made by solution phase methods and converted into
codon 3'-phosphoramidites, with protecting groups that are fully
compatible with automated phosphoramidite DNA synthesis
chemistry. The 3'-hydroxyls were converted into N,N-
diisopropyl-β-cyanoethylphosphoramidites using the general
10 method described above in Examples 1-4. The resulting codon
amidites were single spot by thin layer chromatography (TLC),
and two of the compounds appeared to be more than 90% pure by ^{31}P
NMR.

These individual codon amidites were subjected to
15 several functional tests. First, the individual codons were
used to make a 16-mer dATACTTATCATGAGCC by sequential coupling
onto a nucleoside-bearing, dC-functionalized solid support,
using semi-manual methods. A Biosearch 8750 four-column,
automated DNA synthesizer, running a synthesis program without
20 the coupling step, was used for detritylation, oxidation and
capping, as well as washing between steps.

Coupling was done manually in the following manner.
Under argon, the codon amidite was dissolved in acetonitrile to
make a 100 mg/mL solution, and 100 μL of this solution was taken
25 up in a 1-cc tuberculin syringe (luer slip-tip). Tetrazole was
dissolved in CH$_3$CN to make a 0.6 M solution, of which 100 μL was
taken up in a second syringe.

The DNA synthesizer instrument was paused before the
time that coupling would normally occur, and the liquid was
30 removed from the column by an argon drying step. The column was
removed from the instrument, and one syringe was inserted into
each end of the column. The syringe containing the amidite was
discharged into the column. The excess volume was allowed to
enter the syringe containing the tetrazole solution at the
35 opposite end. Next, the tetrazole-containing syringe was
discharged, and the first syringe was allowed to fill with

excess mixed fluid. The process was repeated three times, and the column was allowed to stand for 1 minute.

After the syringes were removed, the column was placed back on the instrument. Oxidation, capping and detritylation

5 steps were performed as appropriate to complete the desired DNA structure. At the end of the synthesis, the DNA fragment was cleaved from the solid support and, at the same time, the exocyclic amine and internucleotide phosphate protecting groups were removed by treating the DNA product with concentrated

10 NH_4OH for 72 hours at room temperature. Oligopac reverse phase cartridges (commercially available from Millipore Corp.) and the associated protocols for these cartridges were used for purification. On a 0.2 μM synthesis scale, 2 O.D. units (260 nm) of DNA were obtained after the cartridge purification

15 procedure.

The same sequence was then made with monomers and, when tested by co-elution on SAX HPLC, the DNA fragment obtained appeared to be identical to the sequence made with monomers. See Figures 1A, 1B and 1C. The details of the SAX HPLC analysis

20 are as follows: A 5 X 250 mm column of Whatman Partisil-10 was used. Buffer A, 0.002 M phosphate in 20% CH_3CN at pH 6.8, and Buffer B, 0.2 M phosphate in 20% CH_3CN at pH 6.8, were both prepared. The gradient used was 0-75% Buffer B over a 30-minute period, at a flow rate of 1.5 ml/min. The product was

25 detected at 260 nm.

Snake venom phosphodiesterase/calf alkaline phosphatase digests of both 16-mers also looked identical, further establishing the structural integrity of the DNA fragments made by the method of the invention.

30 Next, to evaluate the relative coupling efficiency of the codon amidites, a roughly equimolar mixture of five different codons was coupled to a dC- functionalized solid support. Product tetramers made by coupling the codon amidites were quantified by analytical HPLC. The coupling efficiency of

35 the codon amidite mixture averaged 71%, as compared to the 95-99% coupling efficiency observed for monomers.

The results of co-injection with tetramer standards prepared with monomers showed substantial incorporation of four

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out of five trimer codon amidites being tested and confirmed
that the identity of each of the tetramers produced was
consistent with the intended reaction.

5   For calculation of the relative amounts of tetramers
in the HPLC trace, molar extinction coefficients for the
nucleoside monomers were measured in 50% aqueous methanol at 260
nm: dA (13000); dG (11100); T (7550); dC (4700). These values
were used to correct the areas used to represent the mole % of
each species present: CTTC (30.2%); ATCC (46.5%); ATGC (10.0%);
10   and AGCC (13.2%).

Example 7:   Recombinant DNA Methods

The codon amidites were then used to produce a
"scrambled region", in which 15 internal base pairs were
randomized by coupling a mixture of all five codons (dATA, dCTT,
15   dATC, dATG and dAGC) at five sequential sites. These scrambled
regions were flanked by homologous regions made with amidite
monomers. The procedure yielded a pool of 54-mer fragments with
the sequence shown below in Table 1 as "Codon Amidite 1."

TABLE 1: DNA Sequences Used

Name	Sequence
GST-P1 SfiI	5'-CATGCCATGACTCGCGGCCCAGCCGCC-CATGGCATGCCTCCATACACAGTTGTTTA-3'
Codon Amidite 1	5'-CCAGCATTCTGCGGCCGC(XXX)₅GGGGA-GGTTCACGTACTCAGG-3'

25   The DNA was purified on a reverse phase cartridge.
For the reverse phase analysis, a YMC Corp. C-18 column (5 X 250
mm) having a particle size of 5μ was used. Buffer A was 5% MeOH
in 0.001 M TEAA, pH 7.5. Buffer B was 90% MeOH in 0.001 M TEAA,
pH 7.5. The gradient was 0-100% Buffer B over 30 minutes, with
30   product being detected at 260 nm. With 0.2 μM of
support-immobilized initial nucleoside, 5 O.D. units of purified
54-mer were obtained. The oligonucleotide was further purified
by denaturing urea/acrylamide gel electrophoresis.

This resulting 54-mer pool was used as a primer in the
35   PCR amplification of cDNA encoding a human glutathione

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S-transferase. The human glutathione S-transferase P1-1 cDNA in
the expression vector pKXHP1 was used as a PCR template, in
accordance with the procedure of Widersten, _Comprehensive
Summaries of Uppsala Dissertations from the Faculty of Science_
5   _383_, Uppsala ISBN 91-554-9240-8 (1992). As a preliminary step,
to eliminate an internal _SfiI_ site at position 573-585 in the
human P1-1 cDNA, overlap PCR mutagenesis was performed. A
G-to-A substitution at position 582 removed the _SfiI_ site, while
leaving the amino acid sequence unchanged.

10   For randomization of amino acids corresponding to
position 204-210 (INGNGKQ), the following amplification was
performed with the codon amidite primer pool. The PCR reaction
contained 25 pmol each of the primers GST-P1 _SfiI_ and Codon
Amidite 1 (shown above in Table 1), Perkin-Elmer _Taq_ polymerase
15 buffer with $MgCl_2$ added to 2 mM, 10 ng of template pKXHP1, all
four dNTP's at 250 μM each, and 2.5 units of _Taq_ polymerase, in
a final volume of 50 μl.

        Using an Omnigene thermal cycler, the reaction mixes
were put through 25 cycles of denaturation (94°C, 1 minute);
20 annealing (65°C, 1 minute); and extension (72°C, 1 minute);
followed by a final cycle of extension (72°C, 10 minutes). The
reaction product was gel purified, digested with _SfiI_ and _NotI_,
and gel purified once again.

        The digested cDNA pool was subcloned into a phagemid
25 vector by ligating to 20 ng _SfiI/NotI_-restricted pHEN-1 phagemid
vector in a standard ligation reaction. Ligated phagemid DNA
was then electrotransformed into TG-1 by the procedure of
Hoogenboom et al., _Nuc. Acids Res._, _19_, 4133-37 (1991). After
insertion of the mutagenized cDNA pool into the phagemid vector,
30 sixteen transformants were randomly selected from this mini-
library and were sequenced to evaluate the incorporation of the
scrambled codon region. The recombinant clones were sequenced
through the randomized region using the primer CTATGCGGCCCCATTCA
in the dideoxy chain-termination method of Sanger et al., _Proc._
35 _Natl. Acad. Sci. USA_, _74_, 5463-67 (1977).

- 30 -

These clones showed random incorporation of the proper codon sequences at the correct location. Table 2 below shows all of the codon sequences read in the 16 clones.

TABLE 2

Clone	Sequence
1	ATC-ATG-ATG-ATC-CTT
2	ATG-ATC-ATC-ATG-CTT
3	AGC-AGC-ATC-CTT-ATC
4	ATC-ATG-ATC-ATC-ATC
5	AGC-CTT-ATC-ATC
6	ATC-ATC-ATC-CIT
7	ATC-CTT-ATC-ATC
8	ATG-CTT-ATC-CTT
9	AGC-ATC-ATC-CTT
10	CTT-CTT-ATC-AGC
11	AGC-ATC-ATC-ATG-CTT
12	ATG-ATC-CTT-ATC
13	ATC-CTT-ATC-CTT
14	AGC-ATC-CTT-CTT-ATC
15	ATC-ATA-ATC-ATC
16	ATC-ATC-AGC-ATG

Table 3 below lists the frequency and relative amounts of each codon transcript expressed.

Codon Amidite	Transcript Seen	Frequency	Rel. %
ATA	TAT	1	1.4
CTT	AAG	17	24.6
ATC	GAT	34	43.9

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| ATG | CAT | 9 | 13.0 |
| AGC | GCT | 8 | 11.6 |

Thus, the transcribed base sequences in the scrambled region
matched those expected from the codon amidites, with reasonable
5 correlation with the values obtained by the tetramer HPLC
analysis above.

Example 8:   Altering Proportions of Amidites to
                Improve Representation

        In this experiment, the same procedures used in
10 Example 7 were repeated, except that the concentration of the
more reactive codon amidites was lowered in an attempt to make
expression of each codon more equal, based on the frequencies
seen above in Example 7. Four out of 19 randomly selected
clones had full length library regions. The lower expression
15 quality was believed to be a function of lower overall codon
amidite concentration. However, the results did show the
desired increase in dATG and decrease in dATC, establishing that
changing the relative concentration of the codon amidites in the
coupling mixture has a serviceable effect on expression. Taking
20 all of the data together, the expression ratios of codons in the
clones were closely mirrored by the tetramer model coupling.

        The inventors have therefore demonstrated the ability
to construct desirably diversified DNA libraries using pre-
formed triplet codon amidites and conventional phosphoramidite
25 DNA synthesis.

We claim:

1.   A compound of the formula:
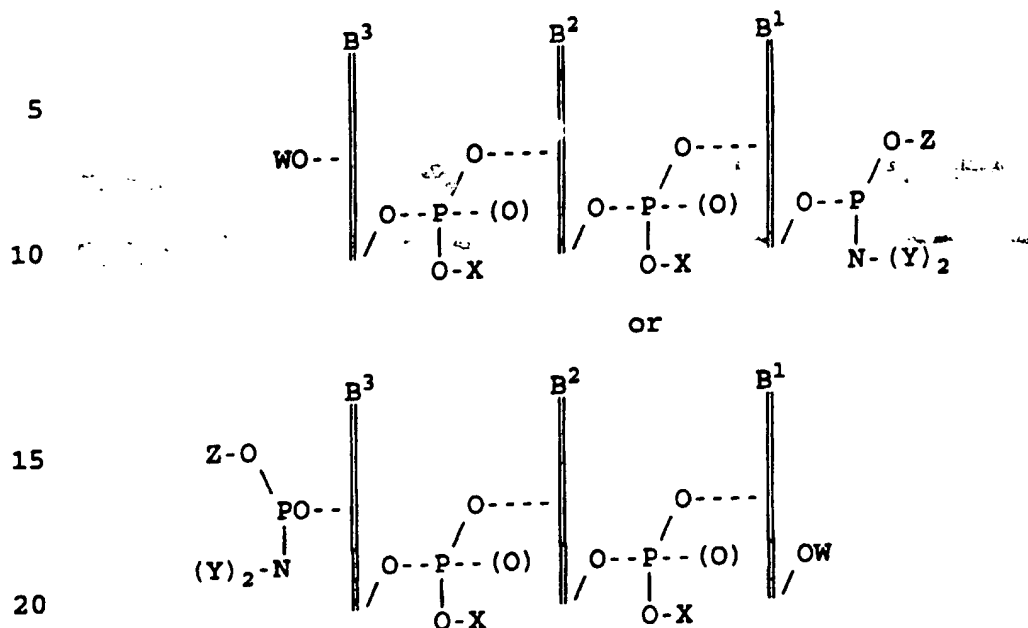
or

wherein W, Y and Z are each independently hydrogen or a
protecting group;

X is -CH$_2$CH$_2$CN;

B^1, B^2 and B^3 are each independently a base selected
from the group consisting of protected adenine,
protected guanine, protected cytosine, protected
or unprotected thymine and protected or
unprotected uracil; and

‖ is the residue of a ribose or deoxyribose.

2.   The compound of claim 1 wherein W is DMT
(dimethoxytrityl); and/or
wherein Y is an isopropyl group; and/or
wherein Z is -CH$_2$CH$_2$CN; and/or
wherein B^1 is selected from the group consisting of
protected adenine, protected guanine, protected
cytosine, and protected or unprotected thymine.

3.   The compound of claim 2 wherein any adenine base
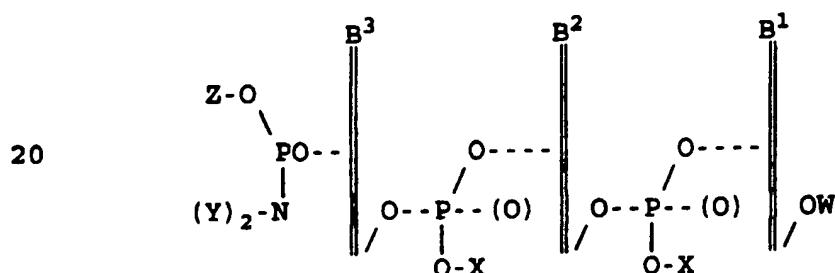is protected with a benzoyl protecting group; and/or

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any cytosine base is protected with a benzoyl
    protecting group; and/or
any guanine base is protected with an isobutyryl
    protecting group.

4. A process for making a compound of the formula:
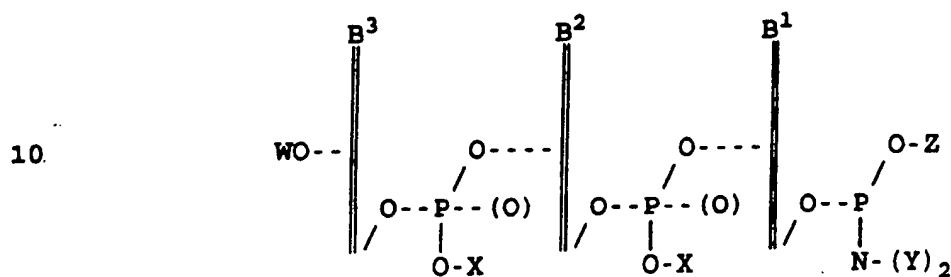
or

wherein W, X, Y and Z are each independently hydrogen or a
    protecting group;
B^1, B^2 and B^3 are independently a base selected from
    the group consisting of protected adenine,
    protected guanine, protected cytosine, protected
    or unprotected thymine and protected or
    unprotected uracil; and
‖ is the residue of a ribose or deoxyribose,
comprising the steps of:
    a.   treating a nucleoside comprising a base, a ribose
       or deoxyribose residue, and a first protecting
       group at the 5' position or the 3' position, with
       a trialkylsilyl halide to produce the
       corresponding 3'- and 5'-substituted nucleoside;

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     b.   removing the first protecting group at the 5' or
3' position to produce a 5' or 3' deprotected, 3'
or 5'-substituted nucleoside;

     c.   coupling the 5' or 3' deprotected, 3' or 5'
substituted nucleoside with a first nucleoside 3'
or 5' phosphoramidite and then oxidizing to form
a phosphate triester;

     d.   deprotecting at the 5'- and 3'-termini to give
the corresponding 3',5'-dihydroxy dinucleoside;

     e.   coupling the dihydroxy dinucleoside with a second
nucleoside 3' or 5' phosphoramidite and oxidizing
to produce the two corresponding 3' or 5' hydroxy
trinucleotides;

     f.   separating away unwanted products; and

     g.   converting the 3'- or 5'-hydroxy trinucleotide to
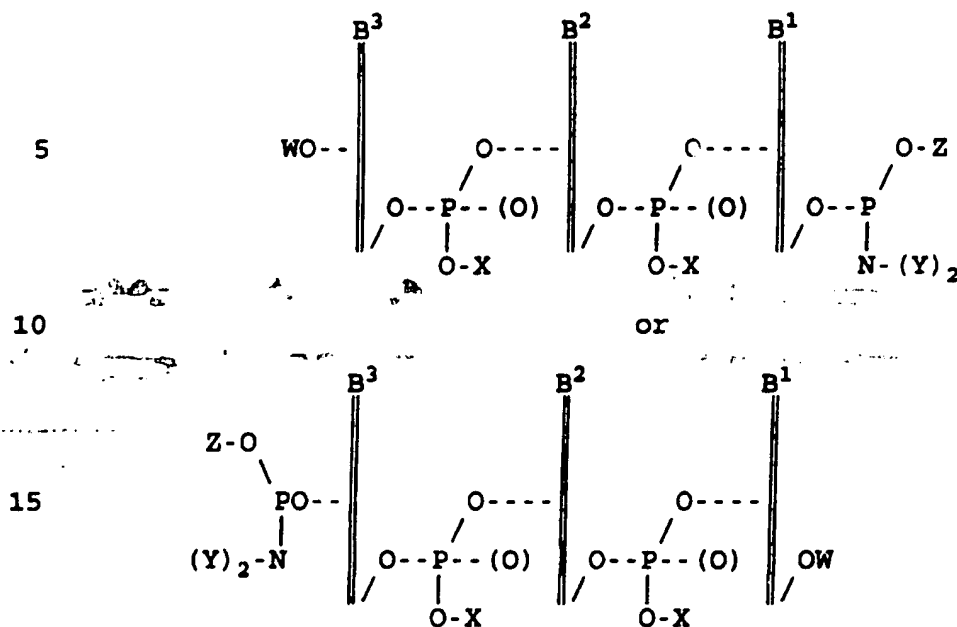a 3'- or 5' phosphoramidite.

     5.   The process of claim 4 wherein said removing step
"b." is conducted under acid conditions; and/or

     wherein, in said removing step "b.", acetonitrile is
used as a solvent; and/or

     wherein, in said removing step "d.", an alcohol or
acetonitrile is used as a solvent; and/or

     wherein, in step "d.", KF is added to the reaction
mixture.

     6.   A panel of from two to twenty 3'- or 5'-
phosphoramidite trinucleotide codons or their complements,
wherein each codon encodes a different amino acid.

     7.   The panel of claim 6 wherein each codon has the
formula:

$$
\begin{array}{ccccc}
B^3 & & B^2 & & B^1 \\
| & & | & & | \\
WO-- & O---- & & O---- & & O-Z \\
& / & & / & & / \\
O--P--(O) & O--P--(O) & O--P \\
/ & | & / & | & / & | \\
& O-X & & O-X & & N-(Y)_2
\end{array}
$$

or

$$
\begin{array}{ccccc}
& B^3 & & B^2 & & B^1 \\
Z-O & | & & | & & | \\
\backslash & & & & \\
PO-- & O---- & & O---- & \\
& / & & / & \\
(Y)_2-N & O--P--(O) & O--P--(O) & OW \\
/ & | & / & | & / \\
& O-X & & O-X
\end{array}
$$

wherein W, X, Y and Z are each independently hydrogen or a
             protecting group;
             B^1, B^2 and B^3 are independently selected from the group
             consisting of protected adenine, protected
             guanine, protected cytosine, protected or
             unprotected thymine and protected or unprotected
             uracil; and
     is the residue of a ribose or deoxyribose.

         8.   The compound of claim 7 wherein W is DMT
(dimethoxytrityl); and/or
             wherein X is $-CH_2CH_2CN$; and/or
             wherein Y is an isopropyl group; and/or
             wherein Z is $-CH_2CH_2CN$.

         9.   A process for synthesizing an oligonucleotide
encoding a sequence of "n" pre-determined amino acids, or its
complement, comprising the steps of:
                 (a)   coupling a trinucleotide codon, or its
                          complement, onto a solid support;
                 (b)   sequentially condensing through a
                          phosphoramidite linkage with each
                          immediately preceding codon, or complement,

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              n-1 trinucleotide codons or their
complements, each codon or its complement
corresponding to the next pre-determined
amino acid in the sequence; and

5   (c)   cleaving the oligonucleotide from the solid
support.

          10. A process of synthesizing an oligonucleotide
encoding a peptide having at least one pre-determined amino acid
position and at least one random amino acid position, comprising

10 the steps of:

          (a)   coupling a first trinucleotide codon, or the
complement to said codon, onto a nucleoside- or
nucleotide-bearing solid support;

          (b)   for each pre-determined amino acid position,

15   sequentially coupling, through a phosphoramidite
linkage, to an immediately preceding codon, or
complement, a trinucleotide codon, or its
complement, corresponding to the pre-determined
amino acid;

20   (c)   for each random amino acid position, coupling
through a phosphoramidite linkage, to an
immediately preceding codon or complement, a
mixture of from two to twenty trinucleotide
codons, at least two of the trinucleotides

25   representing a codon to a different amino acid;
and

          (d)   cleaving said nucleotide from the solid support;
wherein steps (b) and (b) are combined in such a way that each
pre-determined codon sequentially corresponds to each pre-

30 determined amino acid position in said oligonucleotide and
                 wherein the identity and ratio of codons used in
each said mixture are representative of the degree of diversity
desired in the corresponding random amino acid position of said
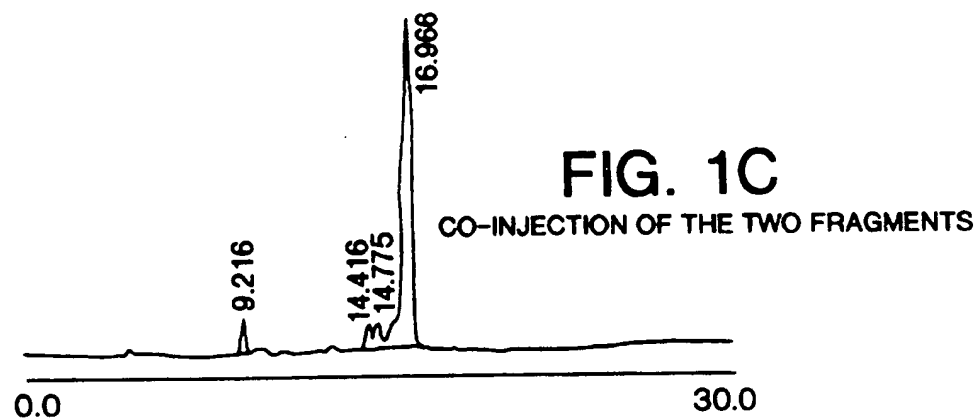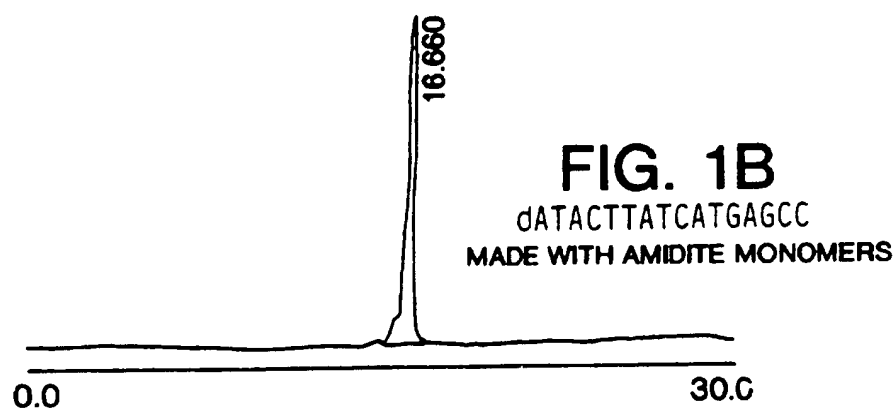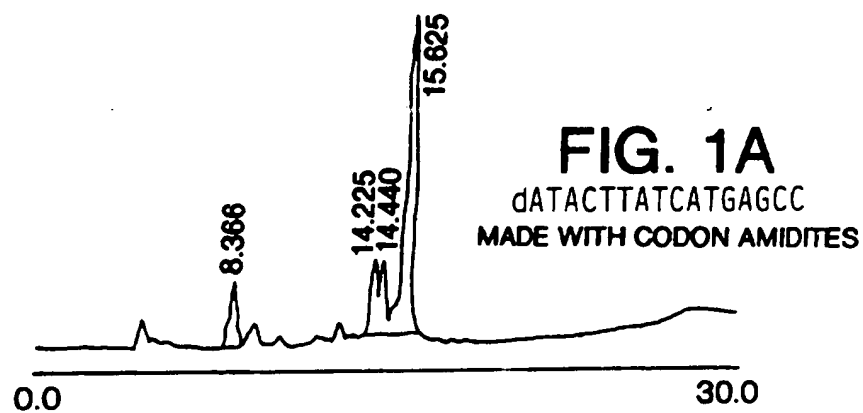oligonucleotide.

11.   A process of synthesizing an oligonucleotide having at least one region of random amino acid positions, of the invention comprises the steps of:

        (a)   coupling at least one pre-existing trinucleotide
5   codon, or complement, onto a nucleoside- or
                nucleotidebearing solid support;

        (b)   for each ˜andom amino acid position, sequentially
                coupling, through a phosphoramidite linkage, to
                the immediately preceding codon, or complement, a
10   mixture of from two to twenty pre-existing
                trinucleotide codons, each codon corresponding to
                a different amino acid; and

        (c)   cleaving the oligonucleotide from the solid
                support;

15 wherein the identity and ratio of codons used in each said mixture are representative of the degree of diversity desired in the corresponding random amino acid position of said oligonucleotide.
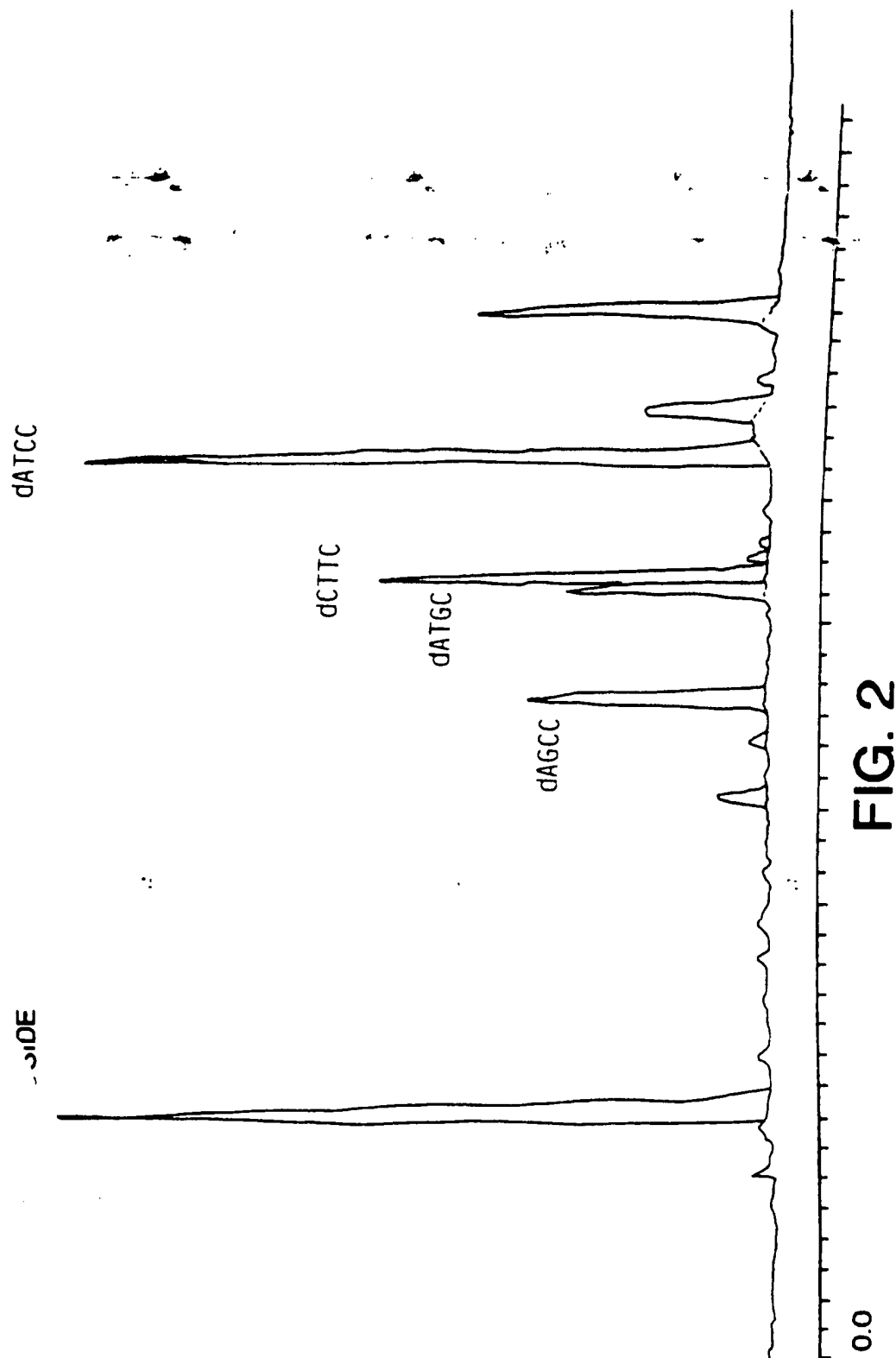
FIG. 1A

dATACTTATCATGAGCC

MADE WITH CODON AMIDITES

8.366   14.225 14.440 15.825

0.0   30.0

FIG. 1B

dATACTTATCATGAGCC

MADE WITH AMIDITE MONOMERS

16.660

0.0   30.0

FIG. 1C

CO-INJECTION OF THE TWO FRAGMENTS

9.216   14.416 14.775 16.968

0.0   30.0

FIG. 2